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Short Communication

Analysis of the Prion Protein in Primates Reveals a New Polymorphism in Codon 226 (Y226F)

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Bovine spongiform encephalopathy has been epizootic in cows for the last two decades, and most probably causes variant Creutzfeldt-Jakob disease in humans. A thorough understanding of prion pathogenesis relies on suitable animal models. Modeling the transmission of BSE to primates is a crucial public health priority, necessary for determining the tissue distribution of the agent and for devising therapies. Susceptibility of humans to BSE is partly determined by polymorphism within the gene encoding the cellular prion protein, *Prnp*, a fact that must be taken into account in primate studies. However, no information is available on *Prnp* polymorphisms in primates. We have sequenced the *Prnp* open reading frames of 30 non-consanguineous Rhesus macaques. All macaques were homozygous for methionine at codon 129, which is polymorphic in humans and seems to modulate prion susceptibility. However, we identified a novel polymorphism in macaque *Prnp*, localized on codon 226 (Y226F). A modulatory effect of this polymorphism on the development of prion disease is possible because codon 226 is close to the suggested binding side of the factor X, which has been invoked as a determinant of the prion species barrier.

Key words: Creutzfeldt-Jakob disease / Polymorphism / Primate / Prions / PrP / *Prnp.*

Prion diseases are transmissible fatal neurodegenerative diseases. The infectious agent is associated with an abnormal isoform of a cellular protein, PrP^{Sc} (Prusiner, 1991). The cellular isoform of the prion protein, PrP^{C}, is a glycosyl-phosphatidyl-inositol-anchored protein of unknown physiological function. Although transgenic mice have been used extensively for the study of prion neuroinvasion (Raeber *et al.*, 1998; Klein *et al.*, 2001), certain aspects of pathogenesis can only be studied in primate models. The macaque model, for example, proved indispensable in the study of new variant Creutzfeldt-Jakob disease (vCJD) (Will *et al.*, 1996). Transmission of bovine spongiform encephalopathy (BSE) to cynomolgus macaques provided the first experimental evidence that BSE is transmissible to a animal model that closely resembles the human situation (Lasmezas *et al.*, 1996) and adapts itself in primates (Lasmezas *et al.*, 2001). The issue of subclinical forms of prion diseases has been raised in a number of studies (Frigg *et al.*, 1999; Hill *et al.*, 2000; Race, 2000). Because primates offer the possibility to acquire tissue at defined time points by performing biopsies, these animals might serve as an important model for studying subclinical prion pathogenesis.

The gene encoding the prion protein (*Prnp*) has been studied in a number of animal species. Functional aspects of PrP^{C} were clarified and important insight into species barrier mechanisms have resulted from these studies (Schatzl *et al.*, 1997; Wopfner *et al.*, 1999). With respect to vCJD the polymorphism of human *PRNP* at codon 129 seems to be of utmost importance. In all vCJD patients, methionine/methionine homozygosity has been found in this position, whereas in caucasian populations the usual distribution pattern is 50% heterozygosity for methionine/valine, 40% homozygosity for methionine/methionine and 10% homozygosity for valine/valine (Windl *et al.*, 1996). It has been proposed that this polymorphism is only present in humans. Indeed, the only mammal that shows an amino acid other than methionine at this position is the Wapiti deer, the gene of which possesses leucine on both alleles (Schatzl *et al.*, 1997). Macaque *Prnp* analysis has been performed on a limited scale in past studies. The currently accepted *Prnp* sequence has been deduced from only 2 Rhesus macaques.

In order to provide a reliable assessment of mutations and allelic variants occurring in macaque *Prnp,* we sequenced the entire open reading frame of 30 Rhesus macaques. The PrP polypeptide is encoded by a single exon, thus permitting PCR amplification of the entire coding region from genomic DNA. In all animals the entire amino acid sequence of mature PrP (residues 23 – 231) could be deduced.

Polymorphisms in *Prnp* occur in all mammalian species. In humans, some of these polymorphisms are associated with spontaneous spongiform encephalo-

pathies (Hsiao *et al.*, 1991; Aguzzi and Weissmann, 1996; Furukawa *et al.*, 1996). One polymorphism that seems to be of utmost importance is the polymorphism at codon 129. In humans this codon is polymorphic, displaying methionine/methionine, valine/valine or methionine/valine (Windl *et al.*, 1996), and seems to influence the susceptibility towards the development of sporadic (Palmer *et al.*, 1991), iatrogenic (Collinge *et al.*, 1991), and new variant Creutzfeldt-Jakob disease. We were not able to detect any polymorphism on this codon in any of our Rhesus macaques. All of the 30 investigated animals were homozygous for the amino acid methionine (methionine/methionine) at codon 129 (Table 1). To exclude that this is caused by genetic drift within a restricted gene pool, the origin of the investigated animals was ascertained. The screened macaques originated from different regions of China, Burma and Central Africa (data not shown) excluding the possibility that the non-existence of the polymorphism on codon 129 could be caused by consanguinity. Previous studies on a limited number of Rhesus macaques and other primates have suggested that codon 129 might be polymorphic in humans but not in animals (Schatzl *et al.*, 1995, 1997). Our study adds further evidence that this is the case. This is reassuring news, since it eliminates the possibility that a polymorphism at this position might lead to difficulties in interpreting past and future prion studies employing Rhesus macaques (Brown *et al.*, 1984; Bons *et al.*, 1999; Lasmezas *et al.*, 2001). Interestingly, codon 129 seems to be polymorphic almost exclusively in humans: the only other species that shows an animo acid other than methionine at this codon is the Wapiti deer, which encodes leucine at both positions (Schatzl *et al.*, 1997).

Interestingly, careful analysis revealed a novel amino acid polymorphism within the macaque *Prnp* gene. This polymorphism was found on codon 226. In the published macaque *Prnp* sequences, this codon contains adenine at position 667 and encodes tyrosine. In 22 out of 30 screened animals we detected adenine on both alleles, encoding tyrosine. One animal was homozygous for thymine, resulting in an amino acid change to phenylalanine, whereas the other 7 macaques showed adenine on one allele encoding tyrosine, and thymine on the other encoding phenylalanine (Table 1; Figure 1 and 2). The calculated gene frequencies were p(Tyr) = 0.85, and q(Phe) = 0.15.

The polymorphism in codon 226 is located at the end

Table 1 Distribution of Polymorphisms at Codon 129 and Codon 226 in the *Prnp* Gene.

	Codon 129		Codon 226
Met/Met	30	Tyr/Tyr	22
Met/Val	0	Tyr/Phe	7
Val/Val	0	Phe/Phe	1

All of the tested macaques were homozygous for methionine on codon 129, while codon 226 is clearly polymorphic. Skin biopsies were used for the isolation of DNA. High molecular weight genomic DNA was extracted using standard protocols. Polymerase chain reactions were performed according to generally accepted protocols (Kwok and Higuchi, 1989). Every PCR included positive and negative controls. Standard conditions for PCR were: 250 ng of genomic DNA, 1 µmol of each primer, 0.25 mM dNTP, 1 mM $MgCl_2$, 1.5 units of *Taq* DNA polymerase (5 U/µl, Qiagen AG, Basle, Switzerland) in a final volume of 25 µl in a commercially available PCR buffer (Qiagen). To prevent evaporation, samples were covered with mineral oil (Sigma). After a first denaturation step of three minutes at 94 °C, samples were subjected to 35 cycles of amplification consisting of 20 seconds at 94 °C, 20 s at 53 °C and 120 s at 72 °C on a Perkin Elmer GeneAmp 9700.

The following primers were used to amplify the complete open reading frame of the prion protein: HM-1 (TGGCGAACCTTGGCTGCTG), KH-13 (CCTCATCCCACTATCAGGAAG). The PCR product was sequenced with the following primers: HM-1, KH-13, CH-7 (TCAGTGGCACAAGCCCAGTA) and CH-8 (GTACTCGTCCGGGGAGTATG). PCR products were separated on a 1.5% agarose gel. PCR fragments were gel-purified (QIAEX II Gel Extraction Kit, Qiagen). Both strands of the isolated DNA were sequenced using the automated ABI 377 sequencing system (Perkin Elmer). Analysis and alignment of the sequences was performed using the ABI Prism software and ClustalW alignment software (www2.ebi.ac.uk/clustalw).

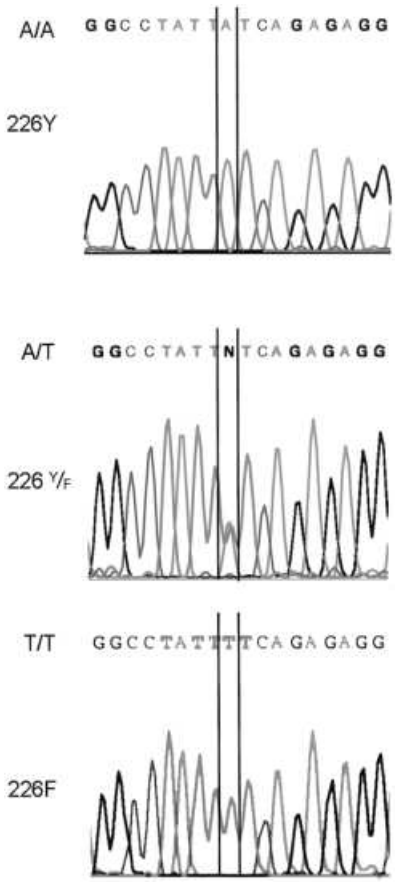

Fig. 1 Y226F Polymorphism.
Adenine on both alleles in position 667 codes for the amino acid tyrosine (Y) (upper panel) while adenine on one allele and thymidine on the other (middle panel) and thymine on both alleles leads to an amino acid change to phenylalanine (F) (lower panel).

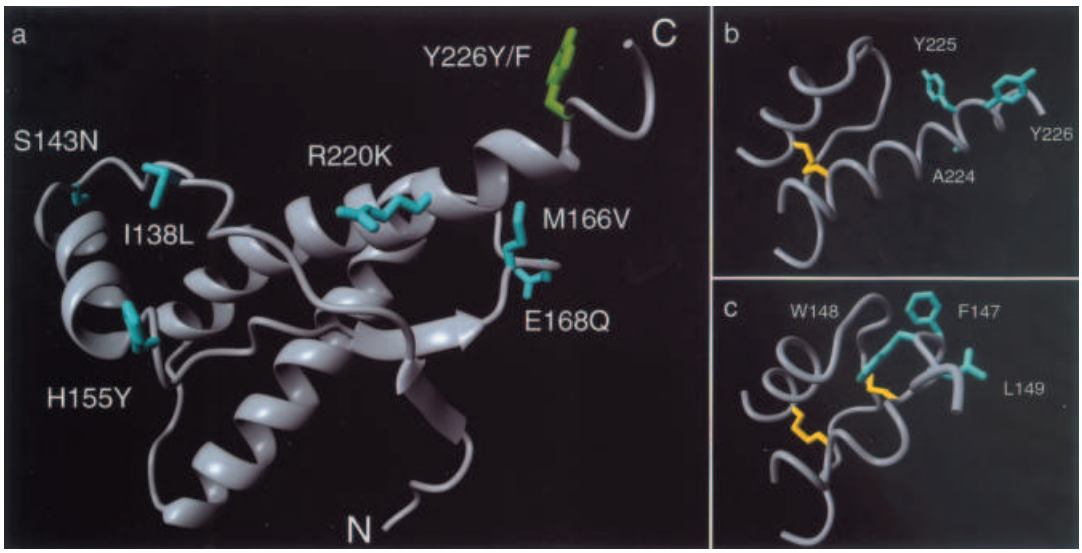

Fig. 2 Three-Dimensional Structure of PrP.
(a) Human PrP fragment 121–230 (hPrP). The protein backbone is shown in grey. Regular secondary structure elements are indicated as ribbons (α-helices) and arrows (β-strands). Amino acid side chains corresponding to species variations between humans and macaques are shown in cyan, and the side chain corresponding to the novel polymorphism in macaques is shown in green. The amino acid types are indicated, and the amino terminus and the carboxy terminus are indicated by 'N' and 'C', respectively. (b) Close-up of the molecular region corresponding to the loop of PrP connecting strand β2, and helix α2, and the carboxy-terminal end of helix α3. (c) Close-up of the molecular region of human Doppel (hDpl) corresponding to the molecular region of hPrP shown in (b). (b and c) The protein backbone is indicated by a spline function through the C^{α}-position of the corresponding amino acids. Disulfide bonds are shown in yellow, and corresponding amino acid side chains between hPrP and hDpl are shown in cyan. The identities of the amino acids are indicated by letters and numbers. The analysis of the 3D structure of human PrP (Protein databank code: AAD46098) was accomplished using the program MOLMOL (Koradi *et al.*, 1996).

of the third α-helix, in the immediate vicinity of the glycophoshatidyl inositol anchor. Whether this newly discovered polymorphism has relevance to the development of prion diseases remains to be investigated. A retrospective study focusing on this polymorphism in macaques that have been used in prion studies could yield interesting insights.

Recent studies on the structure of human and bovine PrP using NMR structure analysis have given valuable information on the three-dimensional structure and structure-functional relationships of the prion protein. As no 3D structure of macaque PrP (mcPrP) is currently available, we asked what influence a Tyr to Phe substitution at position 226 might have on the 3D structure of human prion protein (hPrP) (Zahn *et al.*, 2000), and the conversion of PrP^{C} to PrP^{Sc}. Within the globular domain, there are six amino acid substitutions between hPrP and mcPrP (Figure 2a), corresponding to an amino acid conservation of 95%. All substitutions are located at the surface of the molecule. This suggests that the 3D structures of human and macaque PrP are very similar. In hPrP, the side chain of Y226 is located at the very end of helix α3. Approximately 60% of this residues surface is solvent-accessible, and the closest non-sequential neighboring residue is more that 5 Å away. Thus, it is unlikely that the replacement of a tyrosine to a phenylalanine at position 226, which represents only a minor chemical modification, directly influences the 3D structure of PrP. However, it is noteworthy that this region of PrP is close to the suggested binding side of the proteinaceous factor X (Telling *et al.*, 1995), whose existence has been invoked on the basis of genetic evidence, and which may mediate certain aspects of the species barrier of prion diseases. Thus a modulatory effect of this polymorphism on the development of prion disease is certainly possible, particularly when attempting interspecies transmission of prions.

Interestingly, in the Doppel protein (Dpl), which is the only known protein homologous to PrP, there are similar sequence motives in the C-terminal peptide segment: QAYYQ in hPrP (residues 223–227) and EFWLE in hDpl (residues 146–150). These peptide segments are mostly conserved in the PrP and in Dpl genes studied thus far. Intriguingly, the two aromatic residues (F147 and W148, in Dpl; Y225 and Y226 in PrP) are (i) located at the surface of the molecules and (ii) are evolutionary invariant. These two facts suggest that they serve a specific function. In the 3D structure of human Dpl (hDpl) (Mo *et al.*, 2001), this peptide segment, unlike in hPrP, forms a non-regular secondary structure that folds back onto the loop connecting α2 and β2. This structural dissimilarity is shown in Figure 2, panels b and c. It is conceivable that under certain circumstances a similar conformation might exist in PrP. In this case a Tyr to Phe substitution might have a direct influence on the stability of such a hypothetical state of PrP, and therefore might also influence the formation of PrP^{Sc}.

We searched for further allelic variants occurring in this

population of macaques, and were able to identify a total of 7 allelic variants, all of them silent. A total of four Rhesus macaques showed allelic variants. Animal R12 and animal R31 both carried two common allelic variations in nucleotide 222 and 225. In nucleotide 222 adenosine is changed to a guanine and in nucleotide 225 guanine is changed to adenine. Rhesus macaque R13 showed one additional allelic variation in nucleotide 228: here we could detect a transition of thymine to cytidine. In macaque R27 a cytidine was substituted with a thymine at nucleotide position 748. Rhesus macaque R30 showed three allelic variations, one at nucleotide position 189: here thymine was exchanged for cytidine. Another allelic variation was detected in nucleotide position 261 leading to a change of thymidine to cytidine. Finally, at nucleotide position 270 thymidine was changed to adenosine. Allelic variations found in animal R13 were homozygous, whereas allelic variations in other animals were heterozygous.

Although the functional relevance of silent allelic variants in *Prnp* has not been determined yet (Hunter *et al.*, 1994), the identification of 6 new allelic variants in 4 Rhesus macaques is remarkable, and may be useful in the establishment of genetic risk profiles for the development of prion diseases. Although none of these variants resulted in amino acid changes within PrP, subtle effects on transcription and/or efficiency of translation cannot be excluded. The clustering of these changes in only 4 out of 30 animals prompted us to study the pedigrees of the macaques, yet this analysis did not show first-degree kinship between animals of interest.

The *Prnp* gene residues 60–91 contain an unusual glycine-rich repeat of eight residues (PHGGGWGQ). This sequence is termed octarepeat region. Addition of two, four, five, six, seven, eight or nine octarepeats in humans results in inherited prion disease (Goldfarb *et al.*, 1991; Owen *et al.*, 1992). Deletion of one octareapeat seems to be a protective factor in goats infected with caprine prions (Goldmann *et al.*, 1998). On the other hand, it was shown that lemurs, which carry the shortest octarepeat region known to date (consisting of only two octarepeats), are highly susceptible to BSE prions (Gilch *et al.*, 2000). Rhesus macaques were shown to have 5 octarepeats (Schatzl *et al.*, 1997). We could confirm the presence of 5 octarepeats in 29 out of the 30 studied macaques. In one macaque we could detect a deletion of one octarepeat in one allele. The effect of this deletion upon the susceptibility to prions remains to be elucidated.

This study answers important questions regarding the genetic heterogeneity of the macaque *Prnp* gene. It confirms the hypothesis that codon 129 is not polymorphic in this species. The data presented here have enabled us to formulate a macaque *Prnp* consensus sequence which is based on a sufficient number of individuals. On the other hand, the discovery of a novel polymorphism in macaque *Prnp* (Y226F) poses new questions, and the investigation of the relevance of this polymorphism in the pathogenesis of prion diseases might help to resolve issues like variations of prion susceptibility in primates. Furthermore, these results force a cautionary note on the interpretation of previous primate studies of prion pathogenesis. It is possible that the polymorphism identified in the current study may influence susceptibility to BSE, sCJD and vCJD prions. Therefore, the experimental design of such studies will need to consider these genetic variants and ensure that their impact be duly weighted on the results of such studies.

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